

# Activity of Two Lignin Biosynthesis Enzymes during Development of a Maize Internode

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**Abstract:** To ascertain the temporal relationships of phenylpropanoid and lignin pathway enzymes, phenylalanine ammonia-lyase (PAL) and cinnamyl alcohol-NADPH dehydrogenase (CAD), with lignin concentration, PAL and CAD activities and lignin concentrations were assessed during progressive development of the 10th internode in maize (*Zea mays* L). Enzyme activities were quantified and lignin concentrations were determined by the detergent system of fiber fractionation from lower, middle, and upper sections of the elongating internode harvested 3, 5, 7, 9, 11, 13, and 15 days after the internode had reached 10 mm in length. Over 15 days, a coordinated, sequential, and basipetal pattern of enzyme activities and lignin accumulation evolved through the internode, spanning the stages of rapid elongation, cellular differentiation, and secondary cell wall formation. PAL activity was initiated first, rising to peak activity in elongating tissue, and falling basipetally in tissues as they matured. CAD activity rose in fully elongated, maturing internode tissue where PAL activity was waning following its peak activity. Lignin accumulated in tissues with high CAD activity. CAD activity and lignin deposition patterns were similar: simultaneously increasing in activity and deposition over time while activity and deposition also shifting basipetally through the internode, keeping pace with secondary cell wall formation. Lignin concentration correlated significantly with CAD, but not with PAL.

**Key words:** phenylalanine ammonia-lyase, cinnamyl alcohol-NADPH dehydrogenase, cinnamyl precursors, internode, temporal relationship, acid detergent lignin.

## INTRODUCTION

Lignification during secondary cell wall development indirectly involves the enzymes of the general phenylpropanoid pathway (phenylalanine ammonia-lyase (PAL, EC 4.3.1.5), cinnamate 4-monooxygenase (CMO,

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EC 1.14.13.11), caffeate-*O*-methyl transferase (OMT, EC 2.1.1.68), and 4-coumarate-CoA ligase (4CL, EC 6.2.1.12)), and directly involves the enzymes of the lignin pathway branching from the phenylpropanoid pathway (cinnamoyl-CoA-NADPH reductase (CCR, EC 1.2.1.44), and cinnamyl alcohol-NADPH dehydrogenase (CAD, EC 1.1.1.2)) (Hahlbrock 1977; Grisebach 1981). PAL, which is highly regulated during plant development (Wiermann 1981; Jones 1984; Liang *et al* 1989), converts phenylalanine to (*E*)-cinnamic acid and ammonia. (*E*)-Cinnamic acid is modified during the course of phenylpropanoid metabolism to cinnamyl-CoA precursors used in secondary metabolism path-

ways producing lignins, flavonoids and anthocyanins, phytoalexins, tannins, etc. Evidence suggests that PAL activity does not directly regulate lignin synthesis (Jones 1984; Morrison and Buxton 1993). Neither CAD (or CCR) appear to regulate lignin quality and quantity (Sarni *et al* 1984); however, reduced levels of CAD activity in mutant maize do appear to influence the quality and quantity of the lignin final product (Bucholtz *et al* 1980). CAD reduces cinnamyl aldehydes, produced by action of CCR on cinnamoyl-CoA derivatives, to cinnamyl alcohols that are transported to the cell wall and oxidized to free-radicals that couple, in a random polymerization, to lignin.

In a previous study (Morrison and Buxton 1993), internodes 7 through 14 of maize harvested at the 14th leaf stage were found to be in a range of developmental stages; from primordial in the upper, stalk internodes to fully mature in the lower internodes. Each internode elongated, and underwent differentiation and secondary cell wall thickening, in a basipetal fashion, beginning with the first above-ground internode and proceeding acropetally through the stalk. The basipetal maturation sequence followed through internodes began with PAL activity in elongating internode tissue, then CAD activity in differentiating tissue, and finally with lignin deposition in tissues exhibiting high CAD activity. Lignin deposition correlated positively with CAD activity, but not with PAL activity. The objective of this study was to follow more closely the activities of PAL and CAD and the deposition of lignin during the temporal development of a single maize internode.

## EXPERIMENTAL

### Photo-documentation of internode elongation and lignification

Maize seedlings of single-cross hybrid (Mo17 × B73) were dark pre-germinated in Petri plates containing water-saturated sea sand (product # S25-3, Fisher Scientific, Pittsburgh, PA, USA) under 12 h 28:18°C day: night. Seedlings were transplanted after 3 days to 23 cm diameter pots (one seedling per pot) containing equal parts peat, perlite, sand, and soil, adjusted with iron sulfate to pH 6.3–6.5. Seedlings were grown in a Conviron growth chamber (Model PGW36, Winnipeg, Manitoba, Canada), under 28:18°C day: night, at a 16 h photoperiod, with  $960 \pm 60 \mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetically active radiation (PAR) at canopy height, using fluorescent and incandescent lamps. Seedlings were watered as needed with a solution containing 275 mg liter<sup>-1</sup> N, 60 mg liter<sup>-1</sup> P, and 228 mg liter<sup>-1</sup> K.

When internode 10 (I10), the 5th above-ground internode, reached 10 mm in length, a 5 × 20 mm window

was cut through the sheath of leaf 9 to expose I10. The internode was dotted along its length with Liquitex black acrylic paint (Binney & Smith Inc, Easton, PA, USA) at 1 mm intervals. Internode 10 was photographed every 24 h on ASA 200 35 mm color print film using a Nikon 6006 35 mm camera fitted with a 60 mm micro-lens. A predetermined reproduction ratio (relation between size of image recorded on film and actual subject size) of 1 : 3 was used to develop full-scale photographs on 89 × 127 mm print paper. Measurements of inter-dot spacings on the photographs documented the daily incremental internode elongation. Four plants were photographed and measured in this manner until the internodes ceased elongation.

The basipetal progression of lignification in developing I10 was photo-documented over 13 days. As I10 of similar-stage maize plants reached 10 mm in length, the internode was excised from its stalk, photographed intact, measured for length, cross-sectioned every 10–20 mm (depending on length of internode), and stained with freshly-prepared phloroglucinol-HCl stain (0.2 g phloroglucinol dissolved in 3.3 ml 95% ethanol and 6.7 ml of 36.5% HCl). The cross-sections were photographed and the processed prints appraised for positive (red) staining of cinnamyl aldehydes, markers of lignin (Harris *et al* 1980). One plant was processed every 24 h for 13 days.

### Plant culture for enzyme assays and lignin determination

Maize seedlings of Mo17 × B73 were germinated and potted as above, and grown from 15 June through 5 August 1992 in greenhouse under ambient photoperiod, which averaged  $1465 \pm 60 \mu\text{mol m}^{-2} \text{s}^{-1}$  of photosynthetically active radiation (mid-day at canopy height). Because all seedlings did not begin I10 elongation at the same time, they were grouped into three blocks based on growth stage. Within each block more plants were grown than were used, in order that plants collected for analysis had internodes of very similar length. Temperatures did not fall below 16°C nor exceed 37°C. Seedlings received 275 mg liter<sup>-1</sup> N, 60 mg liter<sup>-1</sup> P, and 228 mg liter<sup>-1</sup> K in solution as needed.

Internode 10 from several plants in each of the three blocks was harvested 3, 5, 7, 9, 11, 13, and 15 days after the internode had reached 10 mm in length. These plants were not paint-dotted. The internodes were excised, divided into equal-length lower, middle and upper section composites, frozen at -20°C, freeze-dried, and finely ground in a sample mill (Udy Corp, Fort Collins, CO, USA). For enzyme assays, 250 mg samples were placed in an ice water bath and sonicated for 45 min in 6 ml of 0.1 M Tris-HCl (product T-3253, Sigma Chemical Co, St Louis, MO, USA) buffer containing 20 mM 2-mercaptoethanol (product M-6250,

Sigma) and 0.5% poly(ethylene)glycol (product P-2139, Sigma), pH 7.6. Samples were stored overnight at 4°C and centrifuged at  $25\,200 \times g$  for 20 min before assay of the supernatants. Lignin content of 0.5 g samples were determined by the procedures of Goering and Van Soest (1970) as modified by Van Soest and Robertson (1985). Lignin was determined to be the weight of sample remaining after cell solubles, hemicellulose, and cellulose had been removed by acid detergent fiber treatment, acid detergent lignin treatment, and high temperature ashing. Analysis of variance was a split-plot design with days of growth assigned to the main plot and location-within-internode to the subplot. Significance of treatment and interaction effects were tested for significance at  $P = 0.05$  and  $0.01$ . Correlation analysis for enzyme assays and lignin content were tested at  $P = 0.05$ .

#### Assay for PAL activity

The deamination of phenylalanine by PAL was monitored according to the method of Abell and Shen (1987). For PAL activity, 2.5 ml of 12 mM phenylalanine in 0.1 M Tris-HCl buffer (pH 8.5) was added as substrate to one set of samples containing 0.5 ml enzyme extract. The PAL controls were a second set of samples containing 2.5 ml of 0.1 M Tris-HCl without phenylalanine. Increase in absorbance due to PAL activity was recorded spectrophotometrically at 290 nm on an LKB Biochrom Ultrospec 4050 spectrophotometer (Cambridge, UK), initially (0 min), and after 60 min of incubation at 30°C. Concentrations of (*E*)-cinnamic acid were calculated by applying the Beer-Lambert law. Total protein concentration was determined by the Bradford method (Bradford 1976) using the Bio-Rad protein assay kit II (product 500-0001, Bio-Rad Laboratories, Richmond, CA, USA). Results were calculated based on total protein concentration.

#### Assay for CAD activity

The activity of CAD was determined by reacting it in enzyme extracts with (*E*)-cinnamaldehyde, to produce cinnamyl alcohol via oxidation of NADPH. The method of Wyrmbik and Grisebach (1975) was used. The reaction mixture (3 ml volume) contained 60 µl of 1.0 mM (*E*)-cinnamaldehyde (product No. 23,9968, Aldrich Fine Chemicals, Milwaukee, WI, USA, made up in 95% ethanol), 600 µl of 1.0 mM NADPH (product N-1630, Sigma), 600 µl of enzyme extract, 50 µl of 20 mM  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , and 1150 µl of 0.1 M  $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$  buffer at pH 7.6. CAD has been shown to be specific for only cinnamyl aldehydes or alcohols, especially substituted ones, in both forward and reverse reactions, with no preference for aliphatic

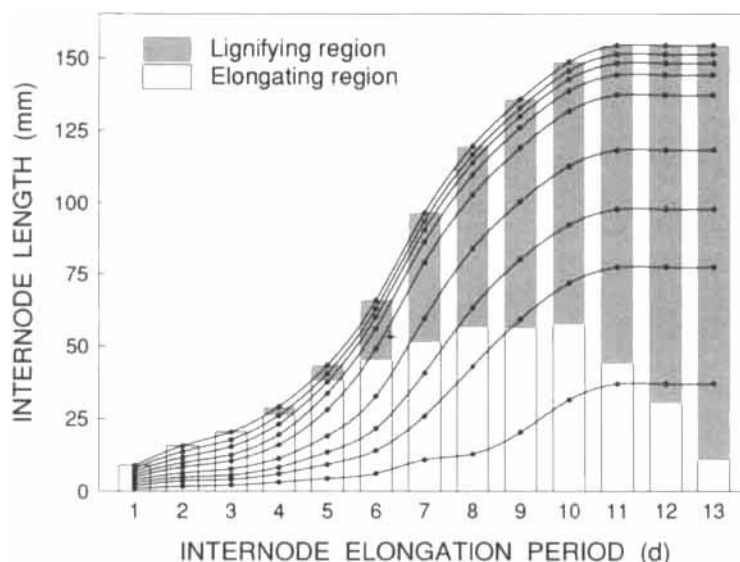
or aromatic alcohols (Mansell *et al* 1974; Wyrmbik and Grisebach 1975). The reduction of aldehyde is favored over the oxidation of alcohol (Sarni *et al* 1984). Because all reaction partners are used or formed in equal amounts ( $\Delta[\text{NADPH}] \cong \Delta[\text{cinnamaldehyde}]$ ), the concentration of cinnamyl alcohol produced could be calculated by the Beer-Lambert law. To account for non-CAD NADPH oxidation, controls were a set of samples containing the reaction mixture without cinnamaldehyde. The oxidation of NADPH caused a linear decrease in absorbance, which was recorded spectrophotometrically at 340 nm initially (0 min), and after 15 min of incubation at 30°C.

## RESULTS AND DISCUSSION

#### Photo-documentation of internode 10 elongation and lignification

The 5th above-ground internode was chosen for observation because it develops vegetatively into one of the longer internodes of the maize stalk. Anatomically, it is the 10th internode of maize, because the first five internodes are compressed and at below-ground level (Kiesselbach 1949; Ritchie *et al* 1989). Internode 10 elongated from 10 to 156 mm in 11 days. The spline plots in Fig 1 shows the daily incremental increase in space between paint dots originally placed 1 mm apart on the 10 mm long exposed internode (day 1 on the graph). Internode tissue elongated sigmoidally over the 11 days in a basipetal direction. Elongation was uniform throughout the internode for the first 3 days, after which elongation activity accelerated in the middle portion of the internode (days 4 through 6), and shifted progressively toward the basal portion of the internode. The shaded-bar plots in Fig 1 show the basipetal progression of lignification, based on phloroglucinol-HCl stain data obtained from individual plants during the 15-day trial. Phloroglucinol-HCl staining, a positive test for lignification, indicates where, in internode tissue, cinnamyl aldehydes accumulate. The heights of the shaded bars indicate the relative accumulation of lignin, and also give an indication as to how soon after cessation of elongation tissue begins to lignify. Tissues of the upper internode stained positively for lignin starting at day 4 and the area staining positively increased basipetally thereafter, indicating that the tissues had ceased elongation, and begun secondary metabolism leading to lignin deposition. By day 13 most of the internode had ceased elongation and begun lignin deposition.

The basipetal wave-pattern of elongation has been studied at the cellular level in oat (*Avena sativa* L) internodes (Kaufman *et al* 1965). During an 11–12 day elongation period, the next-to-last internode in oat owed only one-third of its final length to cell division,



**Fig 1.** Regions of elongation and lignification in developing Internode 10, over 13 days of growth. Spline plots indicate the incremental increase in space between paint dots originally placed 1 mm apart. Shaded bars indicate where lignin has been deposited in the cell wall, as determined by phloroglucinol-HCl stain.

and two-thirds to cell elongation. In dicots, exemplified by the sunflower (*Helianthus annuus* L), internode development was acropetal, the reverse of that in monocotyledons (Garrison 1973). In sunflower, the center of elongation activity began in the basal area, and shifted progressively toward the middle, and later, to the upper end of the internode. The oldest, most developed tissues were in the base of the internode. Hypocotyls of kohlrabi (*Brassica caulorapa* Pasq) and bean (*Phaseolus vulgaris* L) showed the same pattern of development as sunflower; upper segments of hypocotyl continued to elongate after lower segments had ceased elongation and begun differentiation (Havis 1940; Klein and Weisel 1964).

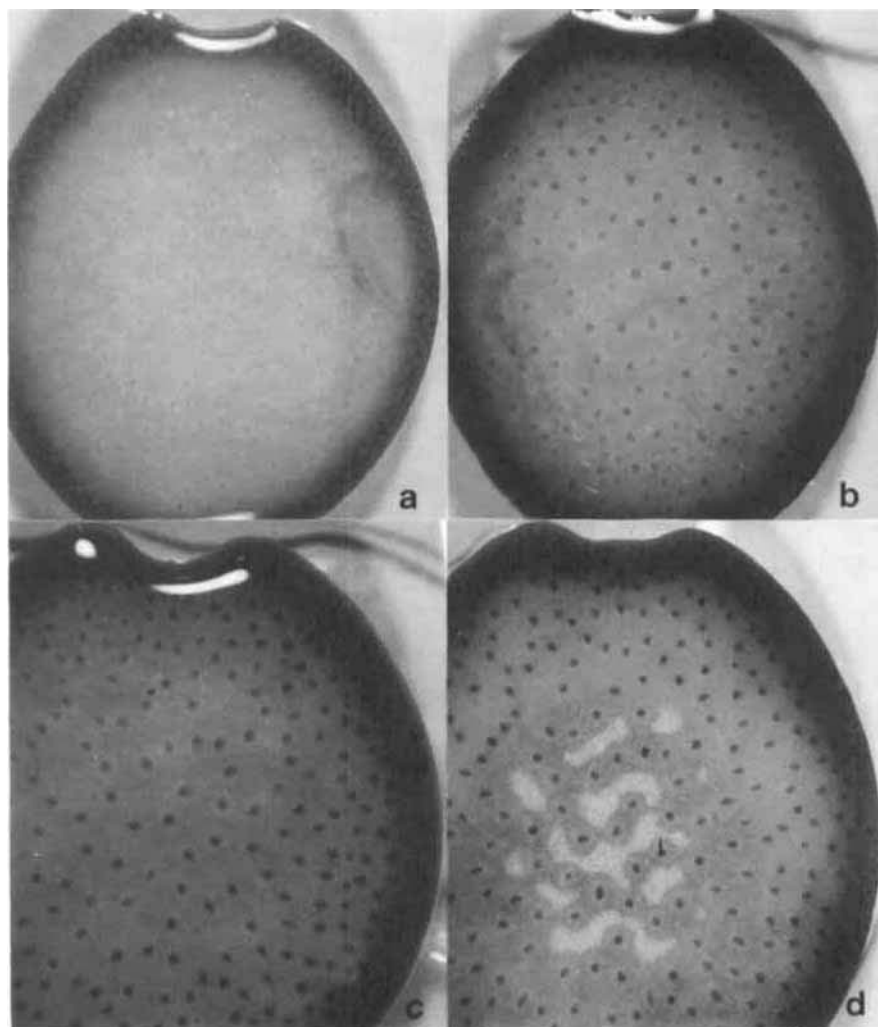
Garrison (1973) found that when 60% of a sunflower internode's final length was attained, the lowermost section had stopped elongation. In our study, maize I10 at the same stage of development (on day 6) had ceased elongation in the uppermost 30 mm and begun secondary cell wall formation (Fig 1). Phloroglucinol-HCl staining indicated the presence of cinnamyl aldehydes in this region, precursors of monolignols that become polymerized as lignin in the cell wall. Peripheral-rind vascular bundles stained first, followed by those in the central pith. Photographs of internode cross-section taken on day 9 of elongation (Figs 2a and b) showed that although vascular bundles of elongating tissue in a lower internode cross-section were lignifying and stained weakly (Fig 2(a)), their bundles were small and less developed compared to the large, well-formed, deeply stained vascular bundles of fully elongated, actively lignifying, upper-internode tissues (Fig 2(b)). By day 13 lower internode vascular tissue were fully developed, and stained heavily (Fig 2(c)). In the upper internode, both vascular and pith parenchyma tissue were

heavily stained (Fig 2(d), darker gray tones in central pith), indicating an accumulation of pith-tissue phenolics that may either cross-link with existing lignin, or be modified by pathway enzymes to monolignols. This tissue also showed cellular lysis.

#### PAL and CAD activity and lignin content

Statistical analysis (split-plot design) of the data indicated that PAL, CAD, and lignin all were significantly different among internode sections. There were differences within internode due to age, and significant internode-section  $\times$  internode-age interaction.

Onset of secondary cell wall deposition, following cell elongation, is marked by an increase in hemicellulose and cellulose deposition and the initiation of lignin synthesis (Bolwell and Northcote 1981). Activity of PAL is initiated, along with other enzymes of secondary cell wall synthesis, as the synthetase activities involved in pectin production wane following primary wall development (Jones 1984). Activity of PAL has been shown to rise in concert with the arabinan and xylan synthetases of hemicellulose formation (Bolwell and Northcote 1981). In support of these facts, our study suggests that PAL activity rises quickly in internode tissue undergoing rapid elongation and differentiation. Its activity begins in the most acropetal (and oldest) tissue of the internode, reaches a peak, then wanes. In Fig 3(a) PAL shows a wave-like rise-peak-wane pattern of activity through the upper > middle > lower sections of the developing internode. The tissues move from elongation to cell differentiation to cell wall maturation phases as PAL activity passes through them. This makes it appear that PAL activity and elongation activity act in close



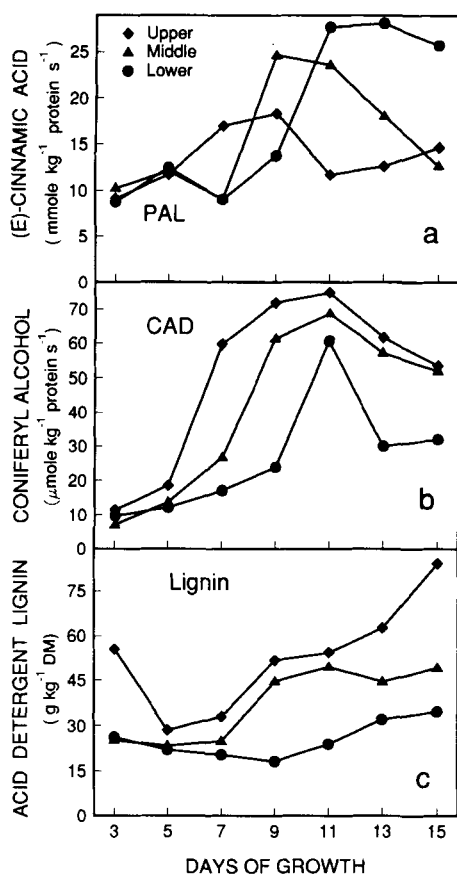
**Fig 2.** (a) Vascular bundles in a cross-section of lower Internode 10 (day 9 of elongation) showed lack of development and weak staining, whereas (b) the vascular bundles of upper internode showed greater development and more intense staining. By day 13 both (c) lower and (d) upper internode cross-sections showed intense staining. Pith parenchyma of upper-internode cross-sections (d) stained positively and exhibited tissue lysis. Original magnification  $\times 4.5$ .

sequence. In Fig 3(a) PALs basipetal activity pattern closely compares with the basipetal retreat of elongation over time, shown in the stack bar (elongating region) of Fig 1. By day 13, elongation is complete in the internode, except for the area near the node, and PAL activity has diminished to base levels in all but the nodal region.

In tissue that lignify, PAL activity is followed in turn by the activity of hydroxylase, transferase, ligase, cinnamoyl reductase, and CAD enzymes of the phenylpropanoid and lignin biosynthesis pathways. CAD activity is associated with tissue undergoing differentiation and secondary cell wall fiber formation. Our results suggest that its activity begins in tissues soon after they cease elongation and are redirected into tissue differentiation by PAL activity. CAD activity initiates in the physiologically advanced tissue of the upper internode, rapidly accelerates to high levels there, and is maintained at high level even as CAD activity extends basipetally into

middle > lower internode section as they too enter into tissue differentiation phase. Figure 3(b) shows that CAD activity increases over time, shifting basipetally through tissues that have ceased elongation and begun maturation processes.

Our study suggests that a slight temporal lag occurs between the disappearance of PAL activity and the appearance of CAD activity. Figures 3(a) and (b) display that, though PAL and CAD activities overlap in the three internode sections, CAD becomes active later than PAL and remains active longer, especially in the upper, oldest tissue. Weissenböck and Sachs (1977), using *Avena sativa* L coleoptiles, noted a similar lag relation between PAL activity and chalcone-flavone isomerase (CFI) activity. They found that PAL activity was greatest in meristem sections of young coleoptiles not yet engaged in flavonoid production, and that PAL activity had waned in older tissue, actively engaged in flavonoid metabolism and showing high CFI activity. Both CFI



**Fig 3.** Activity of (a) phenylalanine ammonia-lyase, (b) cinnamyl alcohol:NADPH dehydrogenase, and (c) lignin concentration in lower, middle, and upper sections of maize internode 10, harvested 3, 5, 7, 9, 11, 13, and 15 days after the internode had reached 10 mm in length.

and CAD, active in maturing tissue, are produced by pathways branching from the phenylpropanoid pathway, and rely on PAL, most active during elongation, for precursors.

CAD catalyzes the reduction of cinnamaldehydes to cinnamyl alcohols, the last enzymatic step in the formation of monolignols which polymerize as lignin. Though CAD is not thought to regulate lignin synthesis, Kutsuki *et al* (1982) suggest that it may have a regulatory role in the formation of guaiacyl and syringyl lignins, and its relative active can influence the quantity and quality (the ratios of *p*-coumaryl and guaiacyl and syringyl monolignols) of the lignin formed. Bucholtz *et al* (1980) found in *Sorghum bicolor* L Moench that a brown midrib-6 mutation reduced the activity of CAD, resulting in less than normal lignin deposition and a three times greater concentration of cinnamaldehyde precursors in the final lignin product. Grand *et al* (1985) noted that the inhibition of CAD activity lead to reduced fluxes of lignin synthesis in poplar (*Populus × euramericana* Dode).

The cinnamyl alcohols formed by action of CAD condense and cross-link by non-enzyme-mediated free-radical polymerization (Hahlbrock 1977; Grisebach

1981). In our study there appears to be no lag-time between the action of CAD and the deposition of lignin (Figs 3(b) and 3(c)). CAD activity and lignin deposition increased over time while progressing basipetally through the internode. Highest lignin deposition was in tissues with highest CAD activity (Figs 3(b) and 3(c)). A significant correlation was found between CAD activity and lignin concentration ( $r = 0.53$ ), though no correlation was found between PAL activity and lignin concentration ( $r = 0.16$ ).

The order of normal plant cell wall fiber formation is hemicellulose > cellulose > lignin. In several forage grasses and legumes Bidlack and Buxton (1992) noted this order of fiber formation and determined that the maximum rate of cellulose deposition followed that of hemicellulose, and maximum rate of lignin deposition followed that of cellulose. Lignin was found to deposit in the cell wall over an extended period, 70 days for lignin in stem segments of orchardgrass (*Dactylis glomerata* L) and smooth brome grass (*Bromus inermis* Leyss), and over 40 days in switchgrass (*Panicum virgatum* L). In our study, lignin was deposited after hemicellulose and cellulose in the normal course of tissue development, and both CAD activity and lignin deposition was protracted. In a previous study Morrison and Buxton (1993) found a strong correlation of CAD activity with hemicellulose, cellulose, and lignin concentrations, indicating that CAD activity coincided with formation of these fibers even though CAD does not directly influence hemicellulose and cellulose formation.

In the study by Morrison and Buxton (1993) eight successive internode of maize were harvested at the 14th-leaf stage and assessed for PAL, TAL, and CAD activity and content of hemicellulose, cellulose, and lignin. At the 14th-leaf stage of development upper internodes are actively elongating, middle internodes are differentiating and beginning cell wall fiber deposition, and lower internodes are in the final stages of maturation, actively engaged in cell wall fiber formation. This study determined that activities of PAL and TAL were highest in the upper, immature internodes and were of progressively reduced activity in the lower, more physiologically advanced internodes. CAD activity was lowest in the immature, upper internode and progressively increased in activity in the more developed, lower internodes. Cell wall hemicellulose, cellulose, and lignin accumulations were low in the upper internodes, and progressively increased through the lower ones, following the same pattern of accumulation as CAD activity. In the present study of PAL and CAD activity and lignin deposition in one internode during its 15 day development period PAL peak activity shifted basipetally to stay in elongating tissue during internode development. CAD activity rose in differentiating tissue undergoing secondary cell wall formation, and lignin content rose in close synchrony with CAD activity. Both studies interpret the sequential and wave-

like relationship between PAL, at the head of phenylpropanoid metabolism, and CAD activity and lignin formation, at the end of lignification, a plant secondary metabolic pathway.

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